EFFECTS OF BORON NUTRITION AND COTYLEDON MANIPULATION ON SYMBIOTIC PERFORMANCE OF BAMBARA GROUNDNUT (*Vigna subterranea L*)

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ABSTRACT

Bambara groundnut was grown in root medium comprising four parts sand and one part perlite under uncontrolled conditions at the National Botanical Institute, Kirstenbosch. The effects of cotyledon removal and boron on the symbiotic performance of this species were investigated. Total phenolics, leaf area, nodulation and levels of nitrogen fixed were determined. Plants grown without boron exhibited lower photosynthetic unit area, reduced concentration of total phenolics, poor nodulation and decreased nitrogen content. Cotyledon removal also led to reduction in leaf area. Unlike boron exclusion, cotyledon removal led to an increase in levels of phenolics and it promoted early nodulation. The levels of nitrogen fixed was however, similar in both experiments (*i.e.* for plants with intact cotyledons and those without cotyledons). The results of this study suggest that boron is essential for symbiotic performance of bambara groundnut and that reducing seedling cotyledon reserves, can lead to delays in nodulation.

INTRODUCTION.

Legumes are an interesting group of plants in that they have their own nitrogen supply that is not immediately available to weeds or other companion crops which might be competing for moisture, mineral nutrients or space (FAO, 1984). Nodulated legumes thus have considerable advantage, particularly in nutrient poor soils. Nonetheless, because rhizobia located inside the nodules provide only nitrogen, legumes must depend on an adequate supply of all other nutrients for optimum growth (FAO, 1984).

Mineral nutrition of leguminous plants have long been debated. These species have been suggested to be nutritionally demanding (Smith, 1982). However there is no experimental evidence that when growing without rhizobia their nutrient requirement is different from that of other plants. An increased requirement for certain elements by nodulated legumes has been reported (Bergersen, 1977), however, they may need some elements not required by non-nodulated legumes. These nutrient elements can affect nitrogen fixation both directly and indirectly (Marschner, 1986).

A specific requirement for molybdenum in N_2-fixing systems has been established (FAO, 1984; Franco, 1978; Marchner, 1986) and its symbiotic function far exceeds its requirement for host plant growth (Robson, 1983). A much higher calcium requirement for root infection and nodule initiation than for root and shoot growth of the host plant has also been reported (Marschner, 1986) and there are indications that greater amount of calcium may be required for nodule function than for growth of the legume host (Smith, 1982).

Boron has been shown to be an essential micronutrient required for normal growth of plant (Marschner, 1986), diatoms (Dugger, 1981), and heterocystous cyanobacteria (Bonilla *et al.* 1990). There has however been considerable disagreement concerning the relative importance of boron uptake by the roots (Raven, 1980). Because its primary mechanism of action is still unknown (Bolanos *et al.*, 1994), the unifying factor seem to be that all life forms that require boron have cell walls, cell wall matrix, or cell envelopes, which are rich in carbohydrates (Lewis, 1980). According to Mclendon's (1976) classification of the origin of mineral nutrient requirements, the requirements for boron are evolutionarily derived.

One of the most rapid responses to boron deficiency is the inhibition or cessation of cell elongation of both primary and lateral roots (Marschner, 1986), leading to bushy and stubby growth.

Several early reviews have also reported that boron deficiency leads to accumulation of phenolic compounds in plants (Dugger, 1983). Phenolics (flavonoids, isoflavonoids and flavones) are of uttermost importance in the legume nitrogen-fixing systems as well as vesicular arbuscular mycorrhizae (VAM) which make phosphorus available to plants. These compounds, released primarily by germinating seeds or young seedling roots of legumes, act as signal molecules to various microorganisms in the rhizosphere (Marschner, 1991). They promote bacterial growth (Hartwig *et al.*, 1991), serve as chemo-attractants (Caetano-Annoles *et al.*, 1988), and induce expression of bacterial nod genes (Long, 1989).

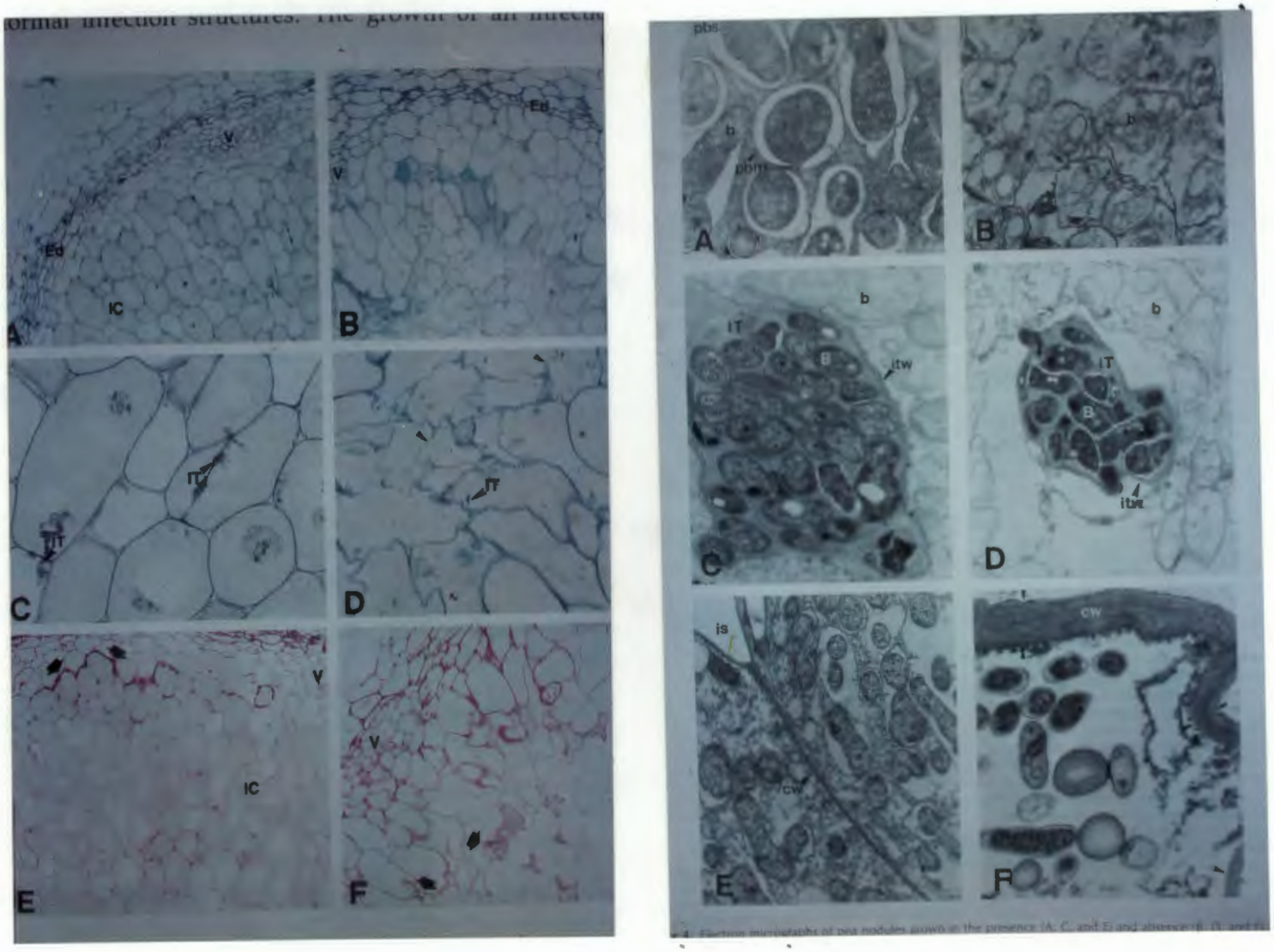
Some studies with boron have shown a direct involvement of this element in nodule development (Pate, 1977; Smith, 1982). Prevention of nodule formation due to boron

deficiency has been reported (Franco, 1977), and where nodules were formed, they lacked vascular strands and bacteroids. The essentiality of this element in heterocystous nitrogen-fixing bacteria was demonstrated by Mateo *et al.* (1986), and for *Pisum sativum*, the absence of boron resulted in a decrease in nodule number (Bolanos *et al.*, 1994). Examination of boron deficient nodules of *Pisum sativum* also showed dramatic changes in cell walls and in both peribacteroid and infection thread membrane, suggesting a role for boron in the stability of these structures (see Figure 1).

Given these effects of nutrient elements on legume symbiosis, this study was undertaken to investigate the effects of boron and cotyledon manipulation on the symbiotic performance of bambara groundnut (*Vigna subterranea).* These effects were assessed through determination of leaf area (an indirect measure of photosynthetic capacity), shoot dry weight, nodule number, nodule dry weight and total nitrogen.

There are several reasons for undertaking this study on bambara groundnut. This legume is one the most important food legumes in Africa. Although it is cultivated throughout Africa, from Senegal to Kenya and from the Sahara to South Africa and Madagascar, bambara groundnut remains neglected by scientific research (NAS, 1979). Yet, fragmentary research results suggest that it is a crop with great promise. The edible grain is rich in essential sulphur containing amino acids such as methionine, cysteine and cystine (NAS, 1979). This species therefore deserves to be taken more seriously as it is one of the two most drought-tolerant cultivated legumes in Africa, the other being Kersting's bean, *Macrotyloma geocarpum* (Kay, 1979). Furthermore, despite the lack of research on bambara, its commercial use in Africa is increasing (NAS, 1979). Improvement of its germplasm for symbiotic nitrogen fixation, high yield, protein content, digestibility as well as the agronomy

of its production deserve intensive study.



A *B*

Magnification?

Figure 1. Light and electron micrographs of pea nodules grown in the presence (A, C, and E) and in the absence (B, D, and F) of boron. *A*: cell walls appeared to be broken in the absence of boron. *B*: symbiosomes have damaged peribacteroid mebranes (pbm) in boron deficient cells and the infection thread wall (itw) and membrane are lost in some sites.

MATERIALS AND METHODS.

Experimental design.

The experiments were carried out in the glasshouse at the National Botanic Institute, Kirtenbosch. Even though the experiment was done in the glass house, the conditions were purely natural because temperature fluctuations and other environmental conditions were not controlled.

Two sets of experiments were done, one in which seedling cotyledons were left intact, and the other in which they were removed. Each experimental set consisted of 12 plastic growth pots.

In both experiments, seeds of Bambara groundnut were grown in a rooting medium comprising four parts sand to one part perlite. At sowing, the seeds were inoculated with *Bradyrhizobium* strain CB 756. Each pot continued to receive 300ml deionised water per day until germination. After germination, seedlings were thinned to three per pot. Where there was poor germination, seedlings were carefully transplanted using forecepts. In one experimental set, cotyledons were clipped off five days after germination. In the other experimental set, seedlings were left with intact cotyledons. In each experiment, six replicates were used, that is the pots were divided into two groups of six, the control and the minus boron treatment. The plants were then supplied with N-free nutrient solutions, in one case

with boron and in the other without boron.

Preparation of nutrient solution.

The preparation was done weekly into thoroughly cleaned 20 litres containers. Before application of nutrient solutions, containers were shaken each time to homogenize the contents. This was done as outlined in Table 1. For minus boron cultures, boric acid was excluded from the solution. Separate beakers were used for each treatment to avoid contamination.

Table 1. Modified Hoagland nutrient solution (Hewitt, 1966).

Reagents	Mol.wt g/mol	stock sln. g/l	dilution ml/20l
Macroelements.			
$MgSO_4.7H_2O$	246.47	246.47	42.6
$CaCl_2$	110.99	111.0	42.6
K_2SO_4	174.27	87.14	42.6
KH_2PO_4	136.09	68.0	21.3
K_2HPO_4	174.18	87.0	21.3
Sequestrene (138 Fe)			
Iron chelate			
(Ciba-Geigy Limited, Base, Switzerland)			
Microelements			
H_3Bo_3	61.83	5.72	21.3
$MnCl_2.4H_2O$	197.91	0.724	21.3
$ZnCl_2$	136.28	0.11	21.3
$CuCl_2.2H_2O$	170.48	0.05	21.3
$Na_2Mo_4.2H_2O$	241.05	0.025	21.3
$CoCl_2.6H_2O$	237.95	0.05	21.3

The plants were harvested after five weeks and separated into nodules, roots and shoots. Shoots and roots were bagged after being flushed with water to get rid of sand.

Leaf area measurements.

Leaves were removed from freshly harvested plants and the leaf area for each single leaf measured with a leaf area meter (Model ...). To obtain average leaf area, the whole plant leaf area was divided by total number of leaves for a ~~per~~ plant.

Assessment of nodulation.

To assess the effects of boron and cotyledon manipulation on nodulation, nodule number was recorded for each treatment at harvest. After that, nodules were removed, oven dried at 70^{0}C for 72 hours and their dry weight measurements taken.

Total phenolic compounds.

Root material which was put in the freezer (-20^{0}C) after nodulation assessment were removed, air-dried for 3 days and ground. Air-drying was preferred to oven-drying in order not to alter the contents of temperature-labile phenolics in the roots. Finely ground root material was put in plastic vials. Prior to extraction, 400 mg of finely ground roots from each experimental treatment were accurately weighed into test tubes. 15 ml 100% HPLC grade methanol was added to the sample in each tube as extractant. The samples were then put in the cold room (0^{0}C) and wrist-shaken from time to time for four days to allow

extraction of phenolics. Assays of total phenolics was done by determining spectrophotochemically the absorbance of each extract at wave-length 350 nm. Absorbance readings were then used to calculate concentration of phenolics in μM daidzein equivalent. The following equation was used:

ϵ = OD/C (Marbry *et al.*, 1970)

where ϵ is the molecular extinction coefficient, (=log 4.04 for daidzein); OD, the optical density (absorbance), and C, the concentration. To obtain the amounts, the concentrations were multiplied by extraction volume and by total root biomass.

Total N determination.

Sample preparation.

Dried samples of both shoots and roots were ground into fine powder and placed in plastic vials. About 250 mg shoot and 150 mg root material were weighed into digestion tubes. The total nitrogen in each sample was converted into ammonium sulphate by Kjeldahl digestion (Bremner, 1965) using selenium catalyst tablets and 10ml concentrated sulphuric acid, after 15 hours of cold digestion.

The following digestion programme was followed:

Step	Temperature (^{0}C)	Time (min)
1	150	40
2	180	20
3	260	60
4	320	120
5	360	180
6	100	999

After this, digestion tubes were removed from the digestion block and made up to 25ml with distilled water. The contents of the tubes were thoroughly mixed with a vortex mixer. Ammonia was recovered by steam distillation (using Markham's still) after addition of alkaline solution (15ml of 50% sodium hydroxide + 2.5% sodium thiosulphate (w/v)). Ammonia gas was trapped in 2 ml of 0.02 N hydrochloric acid, except were samples showed a high ammonia content, in which case 4ml acid was added. The excess was back titrated with 0.005 N solution of sodium hydroxide using Tashiro's indicator. Both distillation and titration were carried out in triplicate. The amount of sodium hydroxide used in back titration was then used to calculate suitable volumes for total N analyses.

Results.

Figure 2 is an illustration of bambara groundnut plants grown in the glasshouse.

Figure 2. Bambara groundnut plants grown in glasshouse at National Botanical Institute, Kirstenbosh.

Leaf area.

Plants with intact cotyledons exhibited larger photosynthetic leaf area compared to those in which cotyledons were removed (Figure 3). Furthermore, the leaves of plants with cotyledons had normal green colour while those without cotyledons looked pale and nitrogen deficient. The effects of boron treatment was different in the two sets of experiments. For plants without cotyledons, the absence of boron led to reduction in leaf area whereas it increased the leaf area in plants with cotyledons (Figure 3). Doesn't look different to me.

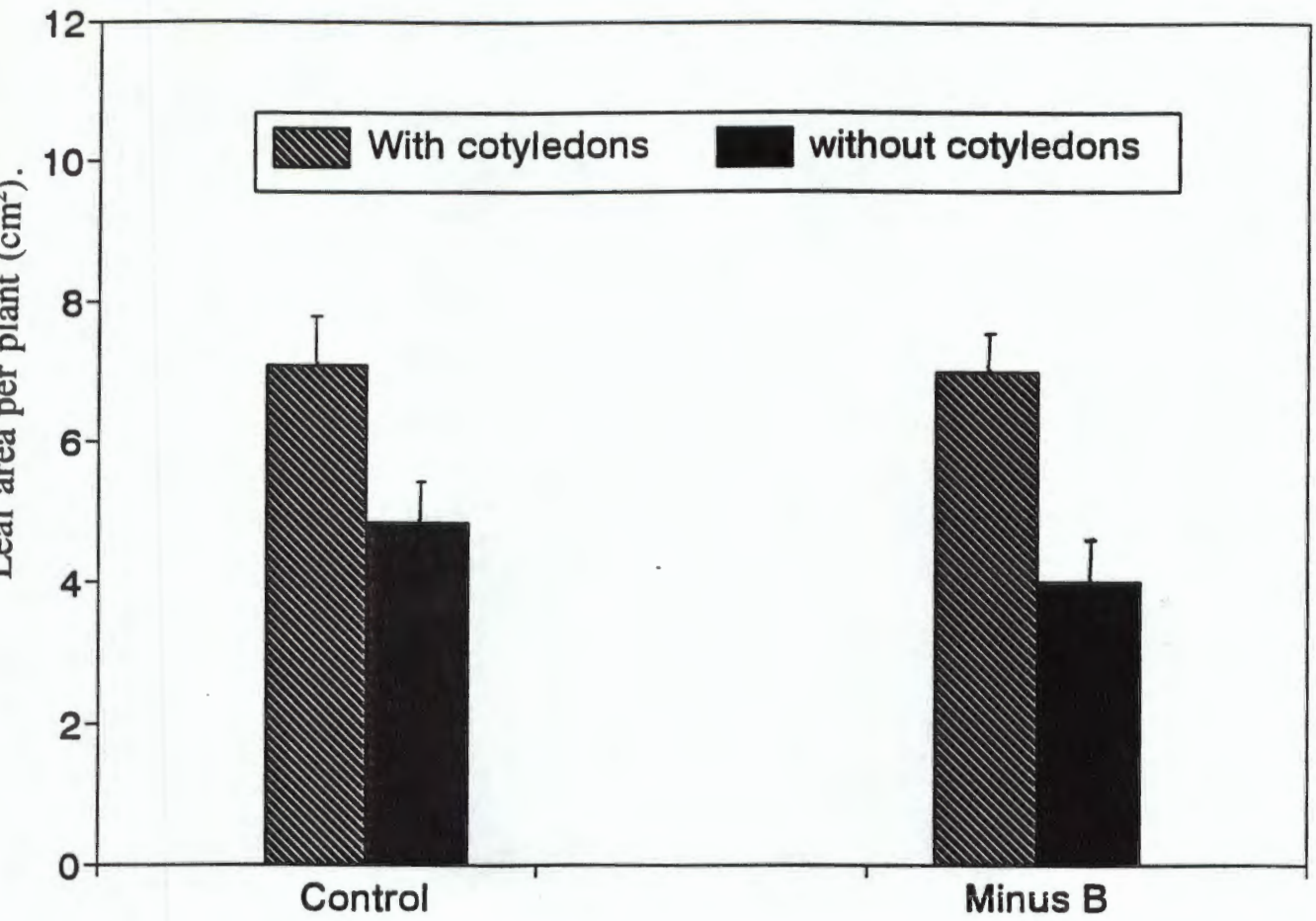


Figure 3. Effects of boron on leaf area. Mean ± SD (n=3 pots).

Nodule formation.

Plants with intact cotyledons showed poor nodulation compared to those whose cotyledons were removed (Figure 4). Nodules of plants with cotyledons were also small in size (data not shown). In both sets (plants with and without cotyledons) the minus boron plants had a low nodule number than the control (Figure 4). About 20% decrease in nodule number was recorded for the minus boron treated plants.

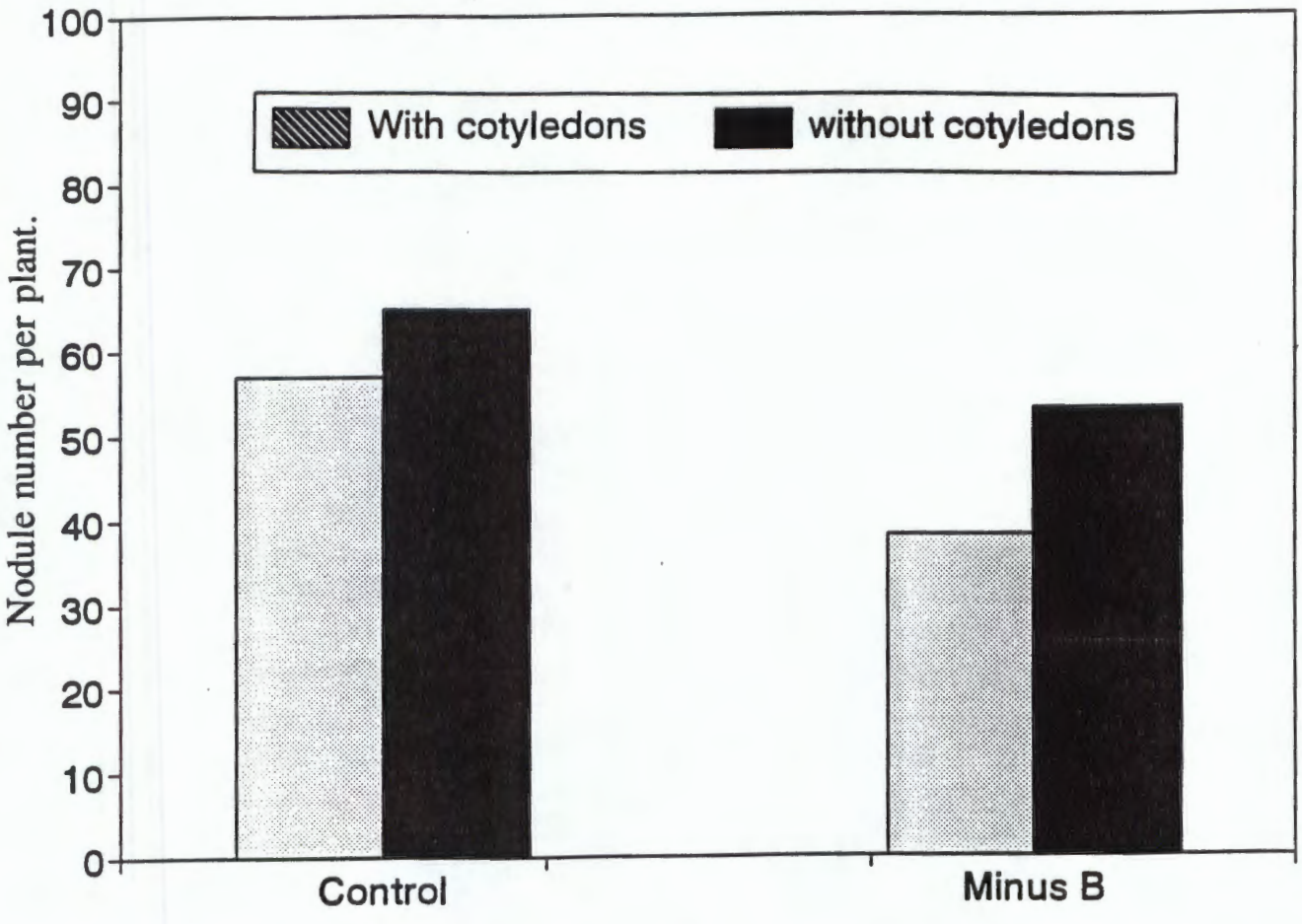


Figure 4. Effects of boron on nodule number. Each value is the average of three replicates.

A corresponding 20% decrease in nodule dry weight was obtained for boron-deprived plants (Figure 5). Nodules formed by boron deficient plants were small in size and they appeared paler compared to those from the control.

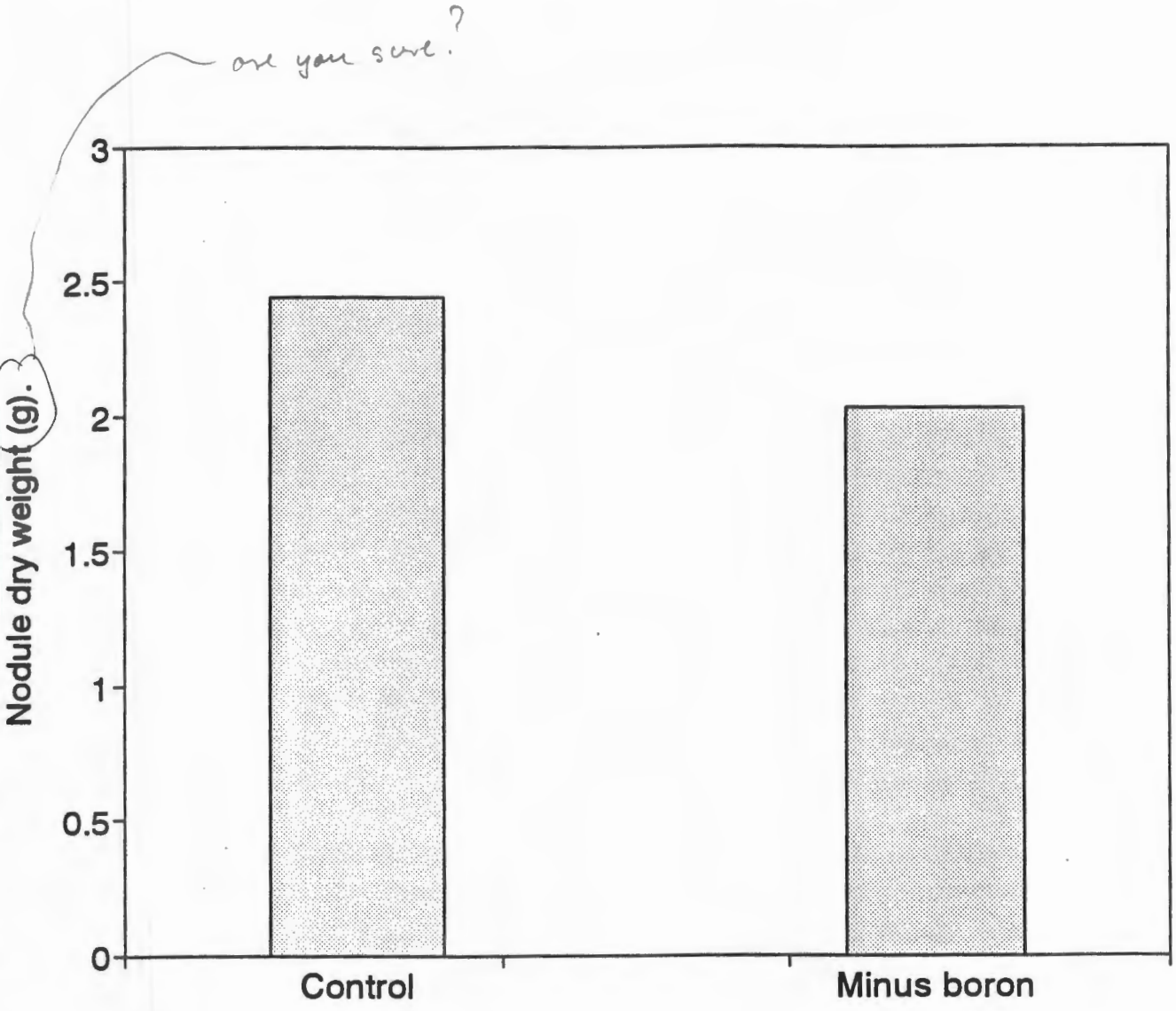


Figure 5. Effects of boron on nodule dry weight. Each value is the average of three replicates.

concentration in μM dadzein equivalent. In both experiments the amount of phenolics released by boron deficient plants was almost 50% lower than that released by boron treated plants (see Figure 6). Comparing plants with and without cotyledons, plants without cotyledons in the control had a high phenolic content while the minus boron plants, both with and without cotyledons showed no difference in their phenolic content (see Figure 6).

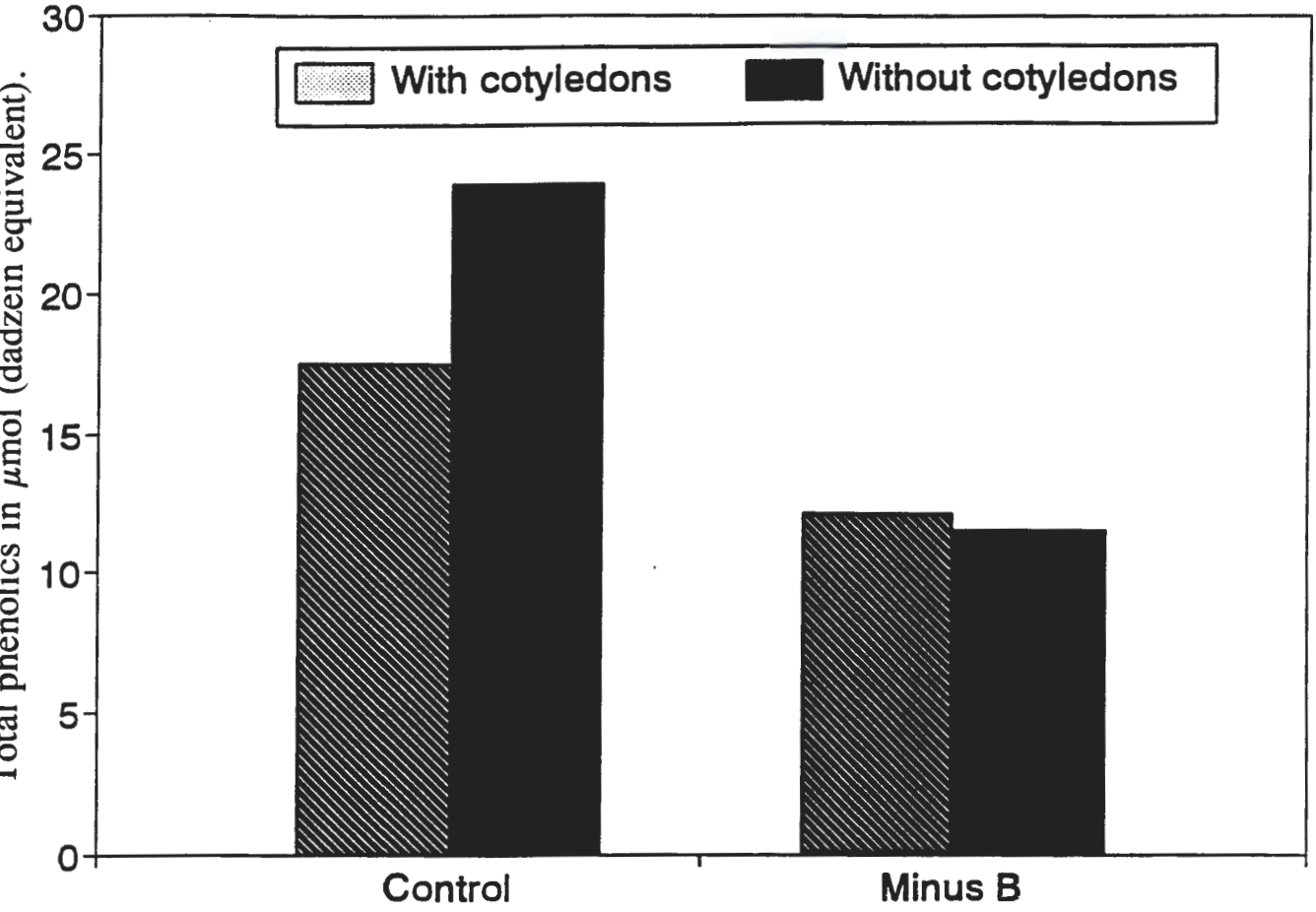


Figure 6. Total phenolics in μM dadzein equivalent. Each value is the average of two replicates.

Total nitrogen.

Total nitrogen content corresponded well with nodule number and nodule dry weight. Exclusion of boron resulted in almost 50% decrease in nitrogen content (Figure 7).

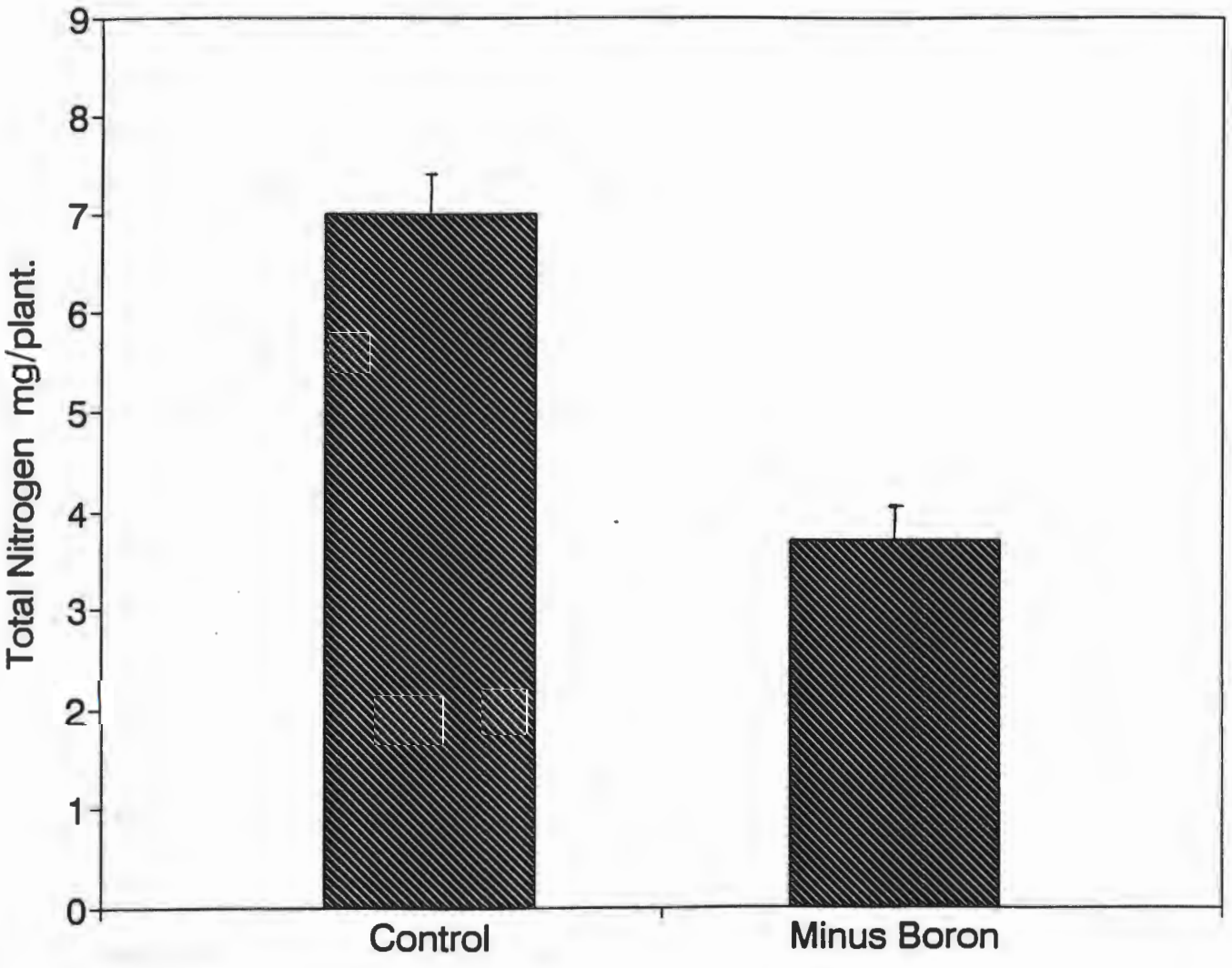


Figure 7. Effects of boron on total nitrogen. Mean ± SD (n=3).

What about total plant biomass & N conc.

Discussion.

Effects of boron on leaf area.

Boron exclusion in this study led to reduction in leaf area (Figure 1). Given that boron is involved in meristematic activity and cell division (Smith, 1982), reduced leaf area would be an expected response to boron deficiency. Furthermore, inhibition of photosynthetic O_2 evolution followed by a decrease in photosynthetic pigment has been shown in *Anabaena* sp.(Mateo *et al.*, 1986). It has also been reported that boron deficient tissues of *Cylindrotheca fusiformis* have lower photosynthetic rates (Dugger, 1983). Leaf photosynthate is the source of energy and carbon skeletons for growth and maintenance as well as bacterial respiration (Vance, 1991). The reduction in leaf area observed in this study may therefore be a consequence of a decrease in photosynthate production which possibly affected leaf expansion. Boron has also been reported to stimulate growth in lower plants such as the nitrogen-fixing cyanobacteria *Nostoc, Muscorum* and *Anabaena cylindrica* (Mateo *et al.*, 1986), suggesting that its absence would limit growth rates in these species.

Effects of boron on total phenolics.

The release of diverse organic compounds by plant roots into the rhizosphere has long been established (Rovira, 1969). However, it is only recently that the functional role of these compounds has started to be determined (Phillips, 1991). This role includes their capacity to induce transcription of nodulation genes in legume-*(Brady)rhizobium* symbiosis (Rolfe and

Gresshoff, 1988; Long, 1989) and to effect chemotacxis towards infectible roothairs (Caetano-Annoles *et al.*, 1988). The results of this study indicate that bambara phenolics, part of which would be nod-gene inducers, tend to be released in higher amounts by boron-treated plants. Thus the poor nodulation and low nitrogen levels in tissues of plants without boron is probably due to the low concentration of nod-gene inducing phenolics. The relationship between concentration of flavonoids and symbiotic indices such as nodule number, nodule dry weight and fixed nitrogen has been reported for alfalfa cv HP 32 by Kapulnik *et al.* (1987). The low concentration of total phenolics in minus boron treatment, however, contradicts the findings that boron deficiency leads to phenolic synthesis (eg Lewis, 1980, Dugger, 1983, Marschner, 1986). This probably suggests species differences since the essentiality of boron in plants is not general (Mateo *et al.*, 1986). The mechanism of distribution of phenolics synthesized in response to boron deficiency in those studies was not reported. According to Dugger (1983) these compounds accumulate in the leaves. It is therefore possible that in bambara these metabolites are not stored in the roots after synthesis.

Effects of boron on symbiotic performance.

The fact that boron is involved in meristematic tissue is suggestive of its role in nodule formation. Nodule number for boron-deficient plants was found to be very low compared to the control. These results are consistent with those of Bolanos *et al.* (1994) who demonstrated a boron requirement for nodulation in *Pisum sativum* (see Figure 1). There are also indications that nodules do not function effectively when formed in the absence of boron

by the observation that boron deficiency symptoms are closely related to those reported for nitrogen starvation. These symptoms include decreased phycobiliprotein and chlorophyll content and the accumulation of carbohydrates (Allen *et al.*, 1969). Interestingly, boron deficiency can be overcome by adding either combined nitrogen or borate (Mateo et al., 1986). It is therefore not surprising that the minus boron plants showed low nitrogen content.

Cotyledon manipulation.

Cotyledon removal is one way of reducing nutrient supply to seedlings, thus forcing the plants to depend entirely on nutrients supplied to the roots. Since all bambara plants were receiving nitrogen-free nutrient solution in this study, reduction in leaf area of plants without cotyledons may be attributed largely to nitrogen deficiency. Plants with intact cotyledons were still being supplimented with nitrogen from seed storage reserves for purposes of growth and development. Although chlorophyll content was not measured, seedling leaves for plants without cotyledons were pale, further suggesting nitrogen deficiency. This condition has been referred to as the "nitrogen hunger" state (Atkins *et al.*, 1989) and is typically exhibited by many legume seedlings when there is insufficient nitrogen supply (FAO, 1984). This is because photosynthesis is dependant on nitrogen for chlorophyll formation and this nutrient is also an important component of photosynthetic enzymes such as Rubisco. The nitrogen hunger state was not observed in plants with cotyledons possibly because bambara is a large-seeded species (NAS, 1979), and thus promotes smoother nutritional transition to full autotrophy in terms of symbiotic nitrogen fixation (Atkins *et al.*, 1989).

Intergrating the results of this study, the effects of cotyledon manipulation on leaf area would alter nitrogen fixation, an indication of the carbon demand of this process. Reduced photosynthetic leaf area would no doubt lead to reduction in photosynthate production and supply, thus causing nitrogen fixation to be carbon-limited (Twary and Heichel, 1991). So the low nitrogen content of plants with reduced photosynthetic leaf area could in part be accounted for by carbohydrate limitation.

Phenolics have been reported to accumulate in legume roots either as phytoalexins (Ward *et al.*, 1974, Rao, 1990, Schmidt *et al.*, 1992), or nod-gene inducers controlling nodule formation (Long, 1989). Factors influencing their release would therefore be injury and/or infection by microsymbiont (Rao, 1990). Although the nod-gene inducing compounds have not yet been identified for bambara, the parallel increase in nodulation and accumulation of root phenolics suggest three things: Firstly, that the presence of boron stimulated increased production of phenolics, secondly that a large proportion of these phenolics probably represent nod-gene inducing compounds, and thirdly, that these nod-gene inducers promoted nodulation in boron-treated plants (Kapulnik *et al.*, 1987; Hungria and Phillip, 1993). Compared ~~to~~ with plants with intact cotyledons, cotyledon removal led to an increase in the level of phenolics in plants receiving boron while in minus boron treatment there was no effect (see Figure 6). When these data on phenolics were related to nodulation, it was clear that control plants showed greater nodulation irrespective of cotyledon manipulation. Intriguingly, the minus boron plants without cotyledons showed lower levels of phenolics (Figure 6), yet had increased nodulation compared to minus boron plants with intact cotyledons (Figure 4), suggesting that a different profile of nod-gene inducing phenolics might have been produced by these wounded plants in the absence of boron.

Conclusion.

The results of this study and the findings by Mateo *et al.* (1986) and Bolanos *et al.* (1994) provide further evidence for the role of boron in N_2-fixation. However, the mechanism of its involvement is still not well understood, and therefore deserves intense study. Also, this study shows that the cotyledon storage reserves are an important nitrogen source for legumes before they start fixing their own nitrogen.

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References.

Allen N. M, and Smith A. J. (1969). Nitrogen chlorosis in blue-green algae. Arch. Microbiol. 69:114-120.

Atkins C. A., Sanford P. J., Dakora F. D., Matthews I. (1989). Nitrogen nutrition of nodules in relation to 'N-hunger' in cowpea (*Vigna unguiculata* L. Walp). Plant Phys. 90: 1644-1649.

Bergersen J. S. (1977). Physiological chemistry of dinitrogen fixation by legumes. *In* A treatise in dinitrogen fixation. eds. Hardy R. W. F., and Silver W. S. pp. 519-556. John-Wiley & Sons. London.

Bolanos L., Estaben E., de Lorenzo C., Fernandez-Pascual M., de Felipe M., Garate A., and Bonilla I. (1994). Essentiality of Boron for Symbiotic Dinitrogen Fixation in Pea (*Pisum sativum) Rhizobium* Nodules. Plant Phys. 104: 85-90.

Bremner J. M. (1965). Total nitrogen. *In* Methods of soil analyses. Part 2. pp. 1147-1178. ed Black C. A. Madeson, Wisk.

Caetano-Anolles G., Christ-Estes D, and Bauer W.D. (1988). Chemotaxis of *Rhizobium meliloti* to plant flavone luteolin requires functional nodulation genes. J. Bacteriol. 170: 3164-3169.

Dugger W. M. (1983). Boron in plant metabolism. *In* Inorganic plant nutrition. eds. Gottingen A. P., and Harvard M. H. Z. pp. 599-627. Springer-Verlag. Berlin.

FAO. (1984). Legume inoculants and their use. FAO. Rome.

Franco A. A. (1978). Micronutrient requirement of legume-*Rhizobium* symbiosis in the tropics. *In* Limitations and potential for biological nitrogen fixation in the tropics. ed. Dobereiner. pp. 161-171. Plenum Press, New York.

Hartwig U.A., Joseph C.M., and Phillips D.A. (1991). Flavonoids released naturally from alfalfa seeds enhance growth rate of *Rhizobium meliloti*. Plant Phys. 95: 797-803.

Hewitt E. J. (1966). Sand and water culture methods used in the study of plant nutrition. *In* technical communication No. 22. Commonwealth Bureau, London.

Hungria M, and Phillips D A. (1993). Effects of a seed colour mutation on *Rhizobial* Nod-Gene_Inducing Flavonoids and Nodulation in the common bean. Molecular Plant-Microbe Interactions 6: 418-422.

Kapulnik Y., Joseph M. C., and Phillips D A. (1987). Flavone limitations to root nodulation and symbiotic nitrogen fixation. Plant Phys. 84: 1193-1196.

Kay D. E. (1979). Food legumes. *In* Crop and Product Digest. Tropical Product Institute, Ministry of overseas Development. pp. 17-25. London UK.

Lewis D.H. (1980). Boron, lignification and origin of vascular plants. New Phytol. 84: 209-229.

Long S.R. (1989). *Rhizobium*-Legume nodulation: Life together in the underground. Cell. 56: 203-214.

Marbry T. J., Markham K. R., and Thomas M. B. (1970). The systematic identification of flavonoids. Springer-Verlag. Berlin.

Marschner H. (1986). Mineral nutrition in higher plants. Academic press. London.

Marschner H. (1991). Root-induced changes in the availability of micronutrients in the rhizosphere. *In* Plant roots - The hidden half. eds. Waisel, Y., Eshel, A., Kafkafki, U. pp. 503-528. Marcel Dekker, Inc. New York.

Mateo P., Bonilla I., Fernandez-Valiente E., Sanchez-Maeso E. (1986). Essentiality of boron for dinitrogen fixation in *Anabaena* sp.PCC 7119. Plant Phys. 81: 17-21.

NAS (1979). Tropical legumes: Resource for the future. NAS. Washington, D.C.

Pate J. S. (1977). Functional biology of dinitrogen fixation by legumes. *In* A treatise in dinitrogen fixation. eds. Hardy R. W. F., and Silver W. S. pp. 473-518. John-Wiley & Sons. London.

Phillips D. A., Maxwell C. A., Hartwig U. A, Joseph C. M., and Wery J. (1991). Rhizosphere flavonoids released by alfalfa. *In* The rhizosphere and plant growth. eds. Keister D. L., and Cregan P.B.

Rao A. S. (1990). Root flavonoids. Botanical Review 56, no.1. pp. 1-84.

Rovira A. D. (1969). Plant root exudates. Bot. Rev. 35: 35-57.

Raven J. A. (1980). Short- and long distance transport of boric acid in plants. New Phytol. 84: 231-240.

Robson A. D. (1983). Mineral nutrition. *In* Nitrogen fixation 3, 37-55. ed. Broughton, W. J.

Rolfe B. G. and Gresshof P. M. (1988). genetic analyses of legume nodule initiation. Annu. Rev Plant Mol Biol. 39: 297-319.

Smith F. W. (1982). Mineral nutrients of legumes. *In* Nitrogen fixation in legumes. ed. Vicent J. M. pp. 155-172. Academic Press.

Schmidt P. E., Parniske M., and Werner D. (1992). Production of phytoalexin glycoceollin I by soybean roots in response to symbiotic and pathogenic infection. Bot. Acta. 105: 18-25.

Smyth D.A., Dugger M.M. (1981). Cellular changes in boron deficient culture of the diatom *Cylinndrothea fusiformis*. Plant Phys. 51: 111-117.

Twary S. N., and Heichel G. H. (1991). Crop physiology metabolism. Carbon cost of dinitrogen fixation assosiated with dry matter accumulation in alfalfa. Crop Sci. 31: 985-992.

Vance C. P. (1991). Root-bacteria interaction. Symbiotic nitrogen fixation.*In* Plant roots. The hidden half. eds. Waisel Y., Eshel A., Kafkafki U. Inc. New York.

Ward E. W. B., Unwin C. H., and Stoessl A. (1974). Experimental control of the late blight of tomatoes with capsidol, the phytoalexin from the peppers. Phytopathology 65:168-169.

Appendix 1.

Absorbance in ODU of total phenolics measured at 350nm.

Treatment	Control	Minus boron.
With cotyledons	0.58	0.58
Without cotyledons	0.61	0.59